LABORATORY METHODS FOR THE DIAGNOSIS OF VARIOLA.

Chalerm Prommas.

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INTRODUCTION.

Whenever an outbreak of small-pox occurs in a community where is imperfectly protected by vaccination, most cases of the disease are usually mild and modified. They are very indefinite; they may be confounded with chicken-pox or other diseases which have similar eruptions. The diagnosis of these cases, even with high experience, can not be clinically made. But if we wait until the cases develop into a typical form, of course, we would be able to recognize them without any difficulty. These cases, from the sanitary standpoint of view, are indeed harmful; they may become dangerous focci and give rise to a malignant assemblage of the disease. It is, therefore necessary, in order to assure the safeguard of the general public, to urge the recognization of the disease by any means as early as possible. During the late war many workers in central Europe, among whom are Paul, Paschen, and others were confronted with this problem. They had devised many laboratory methods for the diagnosis of the obscure cases, some of which had been proved that could be satifactorily used for the purpose.

PAUL'S WORK.

During the last decade Paul has carefully made animal experiments on small-pox and discovered that the variola virus, as well as the vaccine virus after 24 hours of inoculation on a cornea of a rabbit by means of scarification produces absolutely no irritations; the eye is moist and bright as natural. The lines of scratches can barely seen. After

thirty-six hours by the aid of a magnifying glass the inoculated cornea usually be seen covered with tiny, acuminate elevations which look like transparent air bubles. After forty-eight hours those elevations can be seen with naked eyes. In this period the inoculated cornea shows no trace of cloudiness; the conjunctiva is entirely free from inflammation. At 3-4 days, sometimes earlier, the epithelial desquamation begins to take place at the centers of the acuminate elevations. In the further course of development the cornea becomes cloudy and the process finally comes to the cicatrisation.

The recognization of the above manifestations at 2-3 days after the inoculation of the variola virus, on the cornea of the living rabbit is frequently apt to be mistakable due to the epithelial necrosis on the cornea shows little cloudy, being very small and transparent. Sometimes the defect may be caused by traumatism produced in gross scarification, or by other infections. But the manifestations nevertheless can be seen distinctly and definitely when the enucleated eye placed in the sublimated alcohol $\frac{1}{2}$-1 minute. The localized necrosis appear as small round, at times confluent areas, of a chalky whiteness, which stand out sharply against the uniformly milky background. In the centers of the larger pustules, crater-like openings, are often seen. The crater-like pustules are very characteristic for variola and vaccine virus. No ether affections of the rabbit cornea, having similar crater-like necrosis, have ever been described. The inoculation of chicken-pox

material absolutely fails to produce such manifestations. In the case of inflammatory and purulent processes the cloudiness is diffused.

There is slight variation of the manifestations produced in the rabbit cornea by variola and by vaccine virus. The vaccine virus is more aggressive than the variola virus, the former thus produces the epithelial necrosis more rapid and intensive.

In the histological study of the variola rabbit-cornea the epithelial layer at the site of infection has been found raised up like hill (epithelhügel). This according to Paul is specific characteristic for variola and vaccine virus. It is produced by the swelling of the infected epithelial cells through the absorbtion of fluid (hydropsis), not through hyperplasia or well nourishment of the cells, and then the swelling cells push against each other. This tissue change occurs at 36-48 hours after the inoculation. The formation of cell inclusions, due to the pathological increase and alteration of the cell function is also present. In the perinuclear space a round or oval Guarnieri body can be found. At this period the number of Guarnieri bodies produced by the variola virus is usually very few, and sometimes they may not be found at all. But if they are present they are usually located in the central area of the desquamation more than in other locations. It is interesting to note that the vaccine virus produces Guarnieri bodies more abundant and earlier than the variola virus. The nuclei of the individual

epithelial cells show amitotic multiplication without following division of cytoplasm and such cells have been called ''giant cells'' or ''multinuclear cells''.

Hückel following his extensive study of the variola experiment on the rabbit cornea, has recorded as having found box cells (Schachtelzellen) in the infected epithelium usually in the central area of the desquamation. According to his hypothesis the box cells may be the primary stage of the formation of Guarnieri bodies. Later Paul, by the influence of Hückel's work, has restudied and confirmed the discovery of Hückel, except that the box cells were not found in every case. The box cells in the late stage become loose spontaneously and have been found in the smear made from the pustules on the infected rabbit cornea.

The tissue changes produced by the variola virus are identical with those in the human skin as described by Weigert, Unna and Burri.

Based on his discovery, Paul has recommended that the inoculation of the rabbit cornea with suspected material can be used for the diagnosis of obscure cases of small-pox. This method has been tried by many workers such as Hückel, Gins, Weber, and others with satifactory results. Gins also has employed this method to determine as how long the virulence of variola being existed in the nasopharynx of a convalescence. He has found that the positive reaction still could be obtained about four weeks after a recovery.

PAUL'S METHOD.

Both eyes of a rabbit to be inoculated are cocainized

and then scarified with a sterile, fine needle or with a corner of a sterile cover-slip. About four vertical and four horizontal scratches, about 1 m.m. apart, are made on each cornea. The material from a suspected pustule is emulsified with a drop of glycerin or physiological salt solution and then rubbed into the scarified area of the right eye by means of a needle or a platinum loop. The left cornea is not inoculated and is used as a control.

After twenty-four hours the faint outlines of the scratches on both corneas are barely visible and sometimes disappear. After forty-eight hours the control eye should appear normal, while the inoculated cornea, in case of a positive reaction, is covered with tiny, acuminate elevations, which look like air bubles, not opaque, and can not be wiped away with the eyelid. There is no conjunctivitis. If the acuminate elevations appear before this period, or the eye is inflamed, they are produced by traumatism or due to infections other than the variola virus. After thirty-six to forty-eight hours the animal is killed by bleeding after etherization or neck blow. Both eyes are removed with great care that they are not soiled with blood, rinsed in water, and finally transfered to a dish of sublimate alcohol (two volumes of an aqueous saturated solution of corrosive sublimate (boil 2 gms. of corrosive sublimate in 30 c.c. of water, after cooling, filter.) with one volume of 95% alcohol) for about ½-1 minute. In the case of smallpox the inoculated cornea shows small round, at times confluent areas, of chalky whiteness, which stand out sharply

against the uniformly milky background, while the control cornea only becomes uniformly milky. Some of these pustules sometimes may be seen to have crater-like centers. This is pathognomonic of variola. If this characteristic is present according to Paul, the diagnosis is made and no further examination is required.

HISTOLOGICAL EXAMINATION.

In the absence of discreet pustules, a histological examination is necessary. The diagnosis is then ordinarily made either from the presence of the characteristic hill-like elevation of the epithelial layer at the site of infection, or from the finding of Guarnieri bodies and box cells. Guarnieri bodies and box cells are usually not present in great number, and sometimes it is very hard to find them. In this case the diagnosis is sufficiently made on the ground of the presence of the hill-like elevations of the epithelial layer which is the specific tissue change found only in small-pox and vaccine inoculations.

Paul's Rapid Method.

1. After the enucleated eye has been fixed in the sublimate alcohol for at least one hour, the cornea is excised and transfered to 98% alcohol for one minute.

2. Transfer to iodin alcohol which has weak color of iodin, for one minute.

3. One minute in 98% alcohol.

4. One minute in the solution of equal parts of alcohol and chloroform.

5. One minute in pure chloroform.

6. One minute in the saturated solution of hard paraffin in chloroform at 40 C..

7. One minute in melted hard paraffin at 60 C..

8. Embed the cornea with the convex surface down in a salt glass cellar which has been previously smeared with glycerin.

9. Just there is solid film formed on the surface of paraffin, immerse it immediately into cold water. After the paraffin is practically solidified, remove it out from the cellar and cut it into parts with a small, sharp knife, and then cut sections 5 microns thick.

10. Luke water (45 C.) is used for spreading section ribbons. The paraffin sections are picked up with a slide previously smeared with albumin fixative. Moist Joseph paper with absolute alcohol, and squeeze with a thumb on the slide. The slide then is put in the paraffin oven at 60 C. for about from one to two minutes dependent upon the quickness of the sections to adhere to the slide.

11. The sections after having run through xylol, descending alcohols, and water, are stained with hemalum as follows;-

In freshly prepared hemalum (Grübler) , not more than four weeks old, three minutes.

Rinse in water.

Stain with a 1% aqueous solution of safranin five minutes.

Rinse in water.

Differentiate in a 10% solution of tannic acid in water (5-10 minutes).

Rinse in water.

Transfer to 95% alcohol and leave until the color of the epithelium changes to violet.

One minute in Oil of Bergamot.

One minute in xylol.

Mount in balsam.

The Guarnieri bodies will stain pink, while the nuclei of the epithelial cells stain pale violet, and the nuclei of leucocytes stain dark violet.

According to our experience, Paul's Rapid Method did not give us satifactory results, being due to the time is too short; the cornea is not completely dehydrated and the permeation of paraffin is incomplete. We have found that a good result can be obtained from the following technique.

Technique.

1. Fix the enucleated eye ball in the sublimate alcohol for at least one hour.(Other fixing fluids such as Zenker's fluid, Carnoy's fluid, and Bouin's fluid may also be used.)

2. Transfer to 80% alcohol for one hour.

3. Excise the cornea and placed in 95% alcohol for one hour.

4. Transfer to absolute alcohol for one hour.

5. Transfer to chloroform for one hour.

6. Place in chloroform, saturated with soft paraffin at 40 C. for one hour.

7. Transfer to melted soft paraffin at 60 C. for

one hour.

8. Transfer to melted hard paraffin at 60 C. for one hour.

9. Embed, and cut sections about 5 microns thick

Staining Methods.

The sections are stained either with haematoxylin and eosin, or with Giemsa's. The technique of these methods is as follows;-

Haematoxylin Method.

Hemalum and eosin (P.Mayer's);

Reagents required;-

a. Hemalum solution:- 0.1 grm. of hematein dissolved in 5 c.c. of 90% alcohol; add this to 100 c.c. of a 5% solution of potassium alum in distilled water; (using heat to effect the solution of the alum and the hematoxylin). The solution can be used at once after cooling.

b. Eosin solution ;- 0.5% solution of the water soluble eosin in 60% alcohol.

c. Acid alcohol;- 1 c.c. of concentrated hydrochloric acid in 100 c.c. of 70% alcohol.

Technique;- Place sections in xylol to remove paraffin. Run them through absolute alcohol, 95% alcohol, alcoholic Lugol solution, 80%, 70%, and 50% alcohols, one minute in each. Rinse in water.

Stain them in hemalum solution for from one half to one hour.

Rinse in water.

Differentiate in acid alcohol to a reddish brown.

Rinse in water.

Place in 70% alcohol which has been rendered just barely alkaline with sodium hydroxide; leave until the blue color has returned.

Stain with eosin solution one minute.

Run through 80% and 90% alcohols ½ minute in each.

Absolute alcohol one minute.

Xylol one to two minutes.

Mount in balsam.

Giemsa's Method.

Reagents required;

a. Giemsa's solution, the composition of which is;-

Azure II-eosin	3.0
Azure II	0.8
Glycerin	125.0
Methyl alcohol (absol.& neutral)	375.0

b. Alcoholic solution of eosin;- 5 c.c. of a 1% aqueous solution of water soluble eosin plus 100 c.c. of 96% alcohol.

Technique;- The sections are freed from paraffin in xylol.

Absolute, 90%, and 70% alcohols, one minute in each.

Iodin solution (100 c.c. of a 2% solution of potassium iodide plus 3 c.c. of Lugol's solution) ten minutes.

0.2-0.5% aqueous solution of sodium thiosulphate ten minutes.

Wash in distilled water.

Stain with freshly diluted Giemsa solution (1 drop: 1 c.c. of distilled water) ½-1 hour. Replace after 30 minutes with a new portion of freshly prepared solution.

Wash with neutral distilled water.

Blot with filter paper.

Differentiate in the alcoholic solution of eosin.

Absolute alcohol one minute with agitation.

Xylol 1-2 minutes.

Mount in balsam.

SMEAR PREPARATION.

The smear examination of the inoculated rabbit cornea has also been practiced. It requires less time and is simple. The principal features for the diagnosis in the examination of smears are the discovery of Guarnieri bodies and box cells. But as they both are usually scanty, it is rather time consuming to hunt for them, and the success is not always ensured. The elementary bodies such as are found in the smears made from pustules of human variola and varicella, as far as the survey of the literature goes, have never been recorded as having been found in the smears obtained from the variola rabbit cornea.

Technique;- A smear is obtained by pressing and rubbing the surface of a clean slide or a cover-slip on the acuminate elevations of the cornea, and then is fixed and stained. In regards to the fixing and staining methods, various recommendations have been offered by many different

workers; Ewing (1904) recommended to fix the smear with absolute alcohol, and stain with Romanowsky's method; according to Prowazek (1907) it gives more satifactory result if it is fixed in sublimate alcohol; Paul recommends to use the sublimate alcohol for fixing and hemalum for staining.

THE EXAMINATION OF NATIVE PREPARATIONS.

There are three methods that may be employed.

I. Examination of freshly scraped epithelium; Technique:- The corneal epithelium at the site of infection is scraped off with a lancet. Place the material on a slide, to which add lachrymal secretion or a drop of aqueous humor, and a trace of powdered methylene blue. The microscopic examination is made for Guarnieri bodies.

II. Examination of the whole fresh cornea; Hűckel's method:- The inoculated cornea is removed by excising at the limbus by means of curved scissors. Many straight cuts are then made from the periphery to the center of the cornea, by straight scissors. The cornea is carefully spread on a slide on which four small, melted pieces of wax have been previously placed. Place a cover-slip over it with slight pressure. Introduce into the space between the slide and the cover-slip a drop of aqueous humor by means of a capillary pipett, and finally seal it with paraffin cream (melting point at 60 C.).

This method furnishes only a rough review of the tissue changes taking place. Detailed pathological changes can not be studied. Guarnieri bodies are often seen as clear

bright, round or oval bodies lying near the nuclei of the epithelial cells.

III. Vital examination of fresh smear;

Technique:- After a smear has been made in the usual way, a drop of aqueous humor is immediately added, before it is dry.

STUDY OF PAUL'S METHOD.

During the second trimester of the school (1923) I have had an opportunity to study with Dr. Charles E. Simon the Paul method. Material from three actual cases of small-pox was obtained from Colorado, and was tested. The results of the tests are as follows;-

Case I. Pustular material from Mrs. I.

January 4, at 4 P.M. the material was inoculated on the rabbit cornea.

January 5, at 4 P.M. the outlines of the scratches could be barely seen; the eye was apparently normal.

January 6, at 4 P.M. the examination of the eye by the aid of a magnifying glass was negative; the animal was killed by bleeding after etherization. Both eyes were removed, rinsed in water and then placed in sublimate alcohol for ½-1 minute. Many bleb-like elevations and crater-like pustules were present on the inoculated cornea; none on the control cornea.

Histological examination:- The hill-like elevation of the epithelial layer, Guarnieri bodies, and multinuclear cells were found.

Diagnosis; positive.

Case II. Pustular material from Mrs.E.C..

January 4, at 4 P.M. the rabbit cornea was inoculated with the material.

January 5, at 4 P.M. the inoculated eye appeared normal.

January 6, at 4 P.M. the inoculated eye was apparently normal; the animal was killed by bleeding after etherization. The inoculated eye after being placed in sublimate alcohol one minute, showed three small bleb-like elevations; no crater-like pustules.

Histological examination:- The epithelial cells at the site of infection were swollen and disarranged; Guarnieri bodies, box cells, and multinuclear cells were discovered.

Diagnosis; positive.

Case III. Pustular material from Mr. H.H..

January 16, at 2.30 P.M. the material was inoculated on the rabbit cornea.

January 17, at 3 P.M. tiny, bright, bleb-like elevations appeared on the lines of the scratches, and could not be wiped away with the eyelid.

January 18, at 3 P.M. the elevations were larger; the animal was killed by bleeding after etherization. After the eye had been placed in sublimate alcohol for one minute, some of the elevations showed crater-like openings.

Histological examination;- The characteristic hill-like elevation of the epithelial layer, Guarnieri bodies, and multinuclear cells were found.

Diagnosis; positive.

For the purpose of ascertaining whether the presence of Guarnieri bodies could be found as early as twenty-four hours after the inoculation, the material from Case III was inoculated on the cornea of another rabbit on January 17, at 3 P.M.. January 18, at 3 P.M. the inoculated eye was apparently normal. The animal was killed in the usual way. No elevations were seen when the eye was placed in sublimate alcohol. Histological examination; at the site of infection the epithelial cells were swollen and little disarranged. A few Guarnieri bodies, and multinuclear cells were found.

To sum up three clinical cases of small-pox have been tested by Paul's method, and all gave positive reactions. 48 hours after the inoculations the characteristic crater pustules, as well as the hill-like elevations of the epithelial layer at the site of infection were obtained in two out of three cases, while box cells were seen in only one case. Guarnieri bodies and multinuclear cells were found in every case, and in one test they both were present 24 hours after the inoculation.

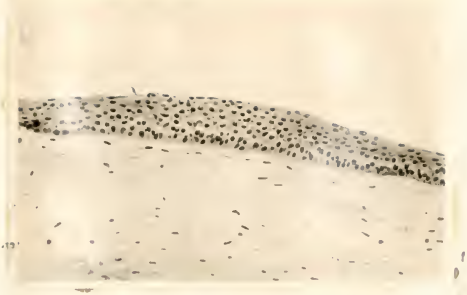

Figure I. Showing the hill-like elevation of the epithelium of the rabbit cornea produced by variola; 48 hours after the inoculation.

Figure II. Showing a multinuclear cell; 24 hours after the inoculation.

IASCHEN'S METHOD.

Iaschen, following his careful study of the series of smears and sections of the skins of various eruptive diseases; variola, varicella, measles, German measles, scarlet fever, lues, herpes zoster, pemphigus neonatorum, pemphigus in adults, has pointed out that the elementary bodies are always found only in the smears of variola and varicella, while in the smears of the rests none have ever been found. In variola the elementary bodies are usually intracellular. If they are seen free, it is ordinarily due to breaking of the epithelial cells caused by bad preparation of a smear. He has also found the elementary bodies in the smears made from the mucous membrane of the nasopharynx of small-pox patients. In the sections of the small-pox skin Guarnieri bodies are usually seen. In varicella the formation of multinuclear cells takes place very early, and thus in the smears of varicella the multinuclear cells are commonly present, while in the smears of variola they are very rare. This is very important characteristic of varicella in the differential diagnosis from variola. He then has emphatically recommended the smear method can be used as a mean for the diagnosis of small-pox.

Technique.

1. Scratch a suspected pustule by means of a corner of a cover-slip.

2. The oozing tissue-fluid is obtained by means of a slight pressure on the pustule, and then smeared on a slide.

3. Dry by air.

4. The slide is placed perpendicularly in a glass of distilled water or saline for 5-15 minutes, old smears require longer.

5. The slide is set up perpendicularly to be dry.

6. Place in absolute alcohol 1-24 hours, or in methyl alcohol 5-15 minutes.

7. Dry by air.

8. Pour on the slide Loffler's mordant (freshly filtered), heat it on a copper plate or by holding over the flame until it steams.

9. Wash thoroughly with distilled water.

10. Stain with Ziehl's carbol fuchsin (undiluted and freshly filtered), heat it until it steams.

11 Wash with distilled water, in the case of overstaining, decolorize in absolute alcohol or in a 5% tannin solution for five minutes, after which wash with distilled water.

12. Dry between filter paper.

Giemsa's method is also recommended by Paschen.

Preparation of Loffler's mordant;-

10 c.c. of a 20% solution of tannic acid is mixed with 5 c.c. of an aqueous solution of ferrous sulphate, saturated at ordinary temperature; add one c.c. of an aqueous or alcoholic saturated solution of basic fuchsin (or methyl violet, or wool black). The solution can be used at once.

Preparation of Ziehl's carbol fuchsin;-

90 parts of a 5% solution of carbolic acid is mixed with 10 parts of a saturated alcoholic solution of basic fuchsin.

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